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Research Article

RESEARCH ARTICLE

Pre-reproductive stress and fluoxetine treatment in rats affect offspring A-to-I RNA editing, gene expression and social behavior

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Abstract

Adenosine to inosine RNA editing is an epigenetic process that entails site-specific modifications in double-stranded RNA molecules, catalyzed by adenosine deaminases acting on RNA (ADARs). Using the multiplex microfluidic PCR and deep sequencing technique, we recently showed that exposing adolescent female rats to chronic unpredictable stress before reproduction affects editing in the prefrontal cortex and amygdala of their newborn offspring, particularly at the serotonin receptor 5-HT2c (encoded by *Htr2c*). Here, we used the same technique to determine whether post-stress, pre-reproductive maternal treatment with fluoxetine (5 mg/kg, 7 days) reverses the effects of stress on editing. We also examined the mRNA expression of ADAR enzymes in these regions, and asked whether social behavior in adult offspring would be altered by maternal exposure to stress and/or fluoxetine. Maternal treatment with fluoxetine altered *Htr2c* editing in offspring amygdala at birth, enhanced the expression of *Htr2c* mRNA and RNA editing enzymes in the prefrontal cortex, and reversed the effects of pre-reproductive stress on *Htr2c* editing in this region. Furthermore, maternal fluoxetine treatment enhanced differences in editing of glutamate receptors between offspring of control and stress-exposed rats, and led to enhanced social preference in adult offspring. Our findings indicate that pre-gestational fluoxetine treatment affects patterns of RNA editing and editing enzyme expression in neonatal offspring brain in a region-specific manner, in interaction with pre-reproductive stress. Overall, these findings imply that fluoxetine treatment affects serotonergic signaling in offspring brain even when treatment is discontinued before gestation, and its effects may depend upon prior exposure to stress.

Key words: RNA editing; fluoxetine; stress; rat; Htr2c; intergenerational

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Introduction

A-to-I RNA editing is a post-transcriptional modification mediated by adenosine deaminases acting on RNA (ADAR) enzymes [1–3]. ADARs bind to double-stranded RNA and convert adenosine (A) to inosine (I), which is read by the translational machinery as a guanosine (G). Occurring at both coding and non-coding regions, ADAR-mediated RNA editing can contribute to translational variability [4] and to the stability and self-regulation of the RNA molecule [5]. The development of high-throughput sequencing-based techniques has enabled the discovery of many novel editing sites in mammals [6].

A-to-I editing occurs in many mammalian tissues, including the brain [7, 8]. Editing changes in coding regions affect key aspects of neurotransmission [8–10]. For example, editing at ionotropic alpha-amino-propionic-acid (AMPA) and kainate (KA) glutamate receptors results in amino acid replacements that lead to significant modifications of channel gating, permeability, trafficking and maturation [11–14]. Another well-studied example of A-to-I editing is the *Htr2c* gene, where editing can occur at each of the five adenosines within the sequence that encodes amino acids 156–160 (sites A–E). Editing at these sites leads to altered encoding of triplet codons resulting in 32 putative isoforms of the G-protein-coupled serotonin receptor [15, 16].

A-to-I editing at mRNA encoding glutamate and serotonin receptors responds to environmental stimulation [17–25]. Using a highly accurate high-throughput targeted approach (microfluidics-based multiplex PCR and deep sequencing—mmPCR-seq [26]), we recently showed that exposing adolescent female rats to chronic unpredictable stress prior to reproduction (prereproductive stress; PRS) affects A-to-I editing in the prefrontal cortex (PFC) and amygdala of their newborn offspring. In particular, editing at the *Htr2c* was affected, resulting in a different pattern of *Htr2c* isoforms in offspring of stress-exposed versus naïve females [22]. We have previously shown that exposure of adolescent females to PRS also results in changes in behavior, stress-related plasma hormone levels, cortical gene expression and neuronal morphology in first- and second-generation offspring [27–31].

Fluoxetine (FLX) is a serotonin-specific reuptake inhibitor (SSRI) commonly prescribed for depression and related affective disorders [32]. FLX treatment affects levels of RNA editing at several sites, including the *Htr2c* and glutamate receptor subunits in culture [33, 34] as well as in the mouse [17, 35–37] and rat [19] brain. Moreover, FLX administration in adulthood reverses the effects of early life stress on RNA editing in the adult mouse brain [35]. Here, we used the mmPCR-seq technique to determine whether post-stress maternal treatment with FLX could reverse the effects of stress on RNA editing in the PFC and amygdala of newborn first-generation (F1) offspring. We also examined the mRNA expression of ADAR enzymes in neonatal PFC and amygdala, and asked whether social behavior in adult offspring would be altered by maternal exposure to stress and/or FLX.

Methods

Animals

Adolescent female Sprague-Dawley rats and adult males were purchased from Envigo (Jerusalem). Housing conditions (except during the stress procedure) included wood-flake bedding, ad lib food and water, 12 h artificial lighting during the day (07–19 h) and temperature maintained at 22 ± 2°C. Animals were randomly distributed across groups (see Experimental procedure below). The number of animals per group appears in the figures. Rats were handled in accordance with the NIH guidelines for the Care and Use of Laboratory Animals, 8th edition [38] and were bred and treated simultaneously to rats described in [22].

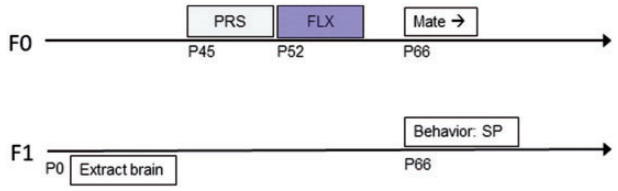

Figure 1: experimental design: intergenerational transmission of PRS/FLX effects

The study was approved by the University of Haifa Committee on animal experimentation (294/13, 351/14).

Experimental Procedure

The experimental procedure is depicted in Fig. 1. Briefly, adolescent [postnatal day (P)45] female rats were group housed (4–6 rats per cage) in 56 × 35 × 19 cm cages. Cages were randomly divided into control (C) and PRS groups. PRS rats underwent a 7-day chronic unpredictable stress (CUS) procedure as described previously [27–31]. Twenty-four hours later (P52), females from C and PRS groups were injected i.p. with either vehicle (VEH) or FLX (5 mg/kg, injection volume 0.5 ml), for 7 consecutive days. A week later (P66), behaviorally naïve males rat were introduced into a cage with 2 female rats and were removed 7 days later. Female rats were returned to their home cage; pregnancy was verified by weekly weighing. Each pregnant rat was moved to a 37 × 30 × 19 cm cage 7 days prior to parturition.

Randomly selected offspring of control and PRS rats (O-C and O-PRS, respectively) were sacrificed on the day of birth (P0) and their PFC and amygdala were extracted for RNA editing and gene expression analysis. Remaining pups were raised undisturbed until P30, then weaned and raised in same-sex, same-condition groups of 4–6. Adults (P60) male and female rats were tested for social preference (see below). Since the *n*'s for RNA editing analysis did not allow to examine gender effects, and no such effects were detected in the gene expression experiments, data from male and female offspring were pooled together. In the behavioral experiment, data for male and female offspring were analyzed separately.

Brain Removal and Dissection

Rats were sacrificed by decapitation and brains were removed and placed on dry ice. Brains were mounted on a cryostat and bilateral samples from PFC and amygdala from neonatal rats were removed guided by the Atlas of the Neonatal Rat Brain [39] and using 0.5 mm punches. Three punches were taken from the PFC and two punches were taken from the amygdala in each hemisphere. Punches from different rats were treated as individual samples. All samples were immediately placed on dry ice and kept at −80°C until further processing.

RNA Extraction and cDNA Preparation

RNA from brain tissue was extracted as described previously [30, 31]. Dissected brain regions were homogenized in 300 µl of

TRIzol (Invitrogen, Carlsbad, CA) and 5 μl glycogen (Sigma-Aldrich, St Louis, MO), then suspended in a total of 500 μl TRIzol. After adding 100 μl chloroform to allow phase separation by centrifugation (14 000 rpm, 15′, 4°C), 250 μl ispropanol (Sigma Aldrich) was added to the aqueous phase. After 12 h in −20°C, RNA was precipitated by centrifugation (14 000 rpm, 15′). The pellet was washed in 500 μl cold 100% ethanol freshly made and stored in −20°C, recentrifuged (7600 rpm, 5′) and then rewashed in 500 μl cold 75% ethanol, recentrifuged (7600 rpm, 5′) then dried. RNA quantities were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) or Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA). RNA quality assessment and cDNA preparation were done as described previously [22]. The 260:280 nm absorbance ratio was measured to assess RNA quality; samples were excluded if the ratio was outside the range of 1.7–2.0, or if RNA concentration was too low. PureLink®RNA Mini Kit (Ambion) was used to further purify some of these samples. cDNA was prepared using iScript™ Advanced cDNA Synthesis Kit (Bio-Rad, Hercules, CA) or High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) or Quanta (Bioscience, Manchester, UK). cDNA used in the mmPCR-seq experiment were purified with Agencourt® AMPure® XP beads (Beckman Coulter, Brea, CA).

Primer Preparation and mmPCR-seq

Editing sites were selected and primer preparation for mmPCR-seq was performed as described previously [22]. Briefly, we designed 48 pools of 2–3 plex multiplex PCR primers (see [22]) to amplify 146 sites. The sizes of the amplicons ranged from 150 to 350 bp. We loaded cDNA and primer pools into the 48.48 Access Array IFC (Fluidigm) and performed target amplification as described previously [26, 40]. PCR products of each sample were then subjected to a 15-cycle barcode PCR and pooled together. All pools were combined at equal volumes and purified via Agencourt® AMPure® XP beads. The library was sequenced using NextSeq 500 (Illumina, USA) with 76 bp paired-end reads. Paired-end reads were combined and mapped onto the genome (rn4) using BWA samse allowing 9 mismatches per read [41]. We aligned the sequencing reads to a combination of the reference genome and 70 bp exonic sequences surrounding known splicing junctions from available gene models (obtained from the UCSC genome browser). We quantified editing levels as described [22] by dividing the fraction of reads containing a 'G' nucleotide by the total reads at each editing site. Only sites covered by 50 mmPCR-seq reads were included. For each comparison, we excluded editing sites that had less than 3 biological replicates, and samples where >30% of editing sites were missing. Custom scripts used to process data are available upon request.

Cluster Analysis of *Htr2c* Isoforms from mmPCR-seq Data

A-to-I RNA editing of the *Htr2c* gene (Rattus norvegicus 5-hydroxytryptamine serotonin receptor 2C) occurs at 5 sites (A through E) and can result in 32 mRNA variants that translate to 24 protein isoforms. We performed editing cluster analysis as described previously [22, 42]. Briefly, we aligned the reads with samtools mpileup (v0.1.18; s [41]), to get the sequence information per genomic location, keeping the data of the original reads. Using an in-house computer program, we were able to find the editing sites in the *Htr2c* cluster in each read. We used only reads that included all cluster editing sites. For each sample, we summed the different combinations of actual editing locations, and found the percentage from the total number of reads that covered all the locations for each isoform. Isoforms that include the E site were not included in the calculations, since editing was not detected at this site.

Quantitative Real-Time PCR

Some of the samples used for RNA editing assessment were also assessed for mRNA expression of RNA editing enzymes and *Htr2c*. In some cases, additional samples were added for qRT-PCR analysis, since RNA quantities were insufficient for both RNA editing and gene expression studies. Primers (see [22]) were designed using *Primer3* [43] software, and synthesized by Integrated DNA Technologies (Coralville, IA). Primer suitability was determined using standard curve analysis, melting curve analysis and linearity at threshold [44, 45]. Quantitative real-time PCR (qRT-PCR) was performed as described previously [31]. Data analysis was performed on dCt values (Ct values) normalized to the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT). ddCt was calculated relative to the control brain region (amygdala) or group (O-C/VEH). Data is represented as fold change, calculated using the ddCt method [45], with standard error of fold change values [44].

Social Preference

The social preference test is commonly used to assess rodent sociability, and has been modified and conducted as described previously [46, 47]. The arena (40lx70wx30h cm) was divided into 2 unequal compartments by a transparent perforated Plexiglas panel, allowing for intact visual and olfactory cues. The experiment rat was placed in the larger compartment and an unfamiliar con-specific partner rat of the same sex and age was placed in the smaller (40lx15wx30h cm) compartment. Rats were habituated to their respective compartments for 5 min. Twenty-four hours later, the partner rat was placed in the smaller compartment, and an unfamiliar object (plastic, 5 × 5 × 8 h, 11 × 8 × 8 h cm) was placed in the larger compartment, 10 cm diagonally from the corner of the arena. One minute later the experiment rat was placed in the arena for 5 min. Several objects and partner rats were used throughout the experiment, and were counterbalanced between groups. Time spent exploring the partner rat and the object was measured using Ethovision XT10.0 software (Noldus Information Technology, Leeburh, VA). Rats that did not complete 30 s of total exploration time (4 rats in total, 1 from each group) were excluded from the experiment.

Statistical Analyses

Data were analyzed with SPSS 23 Statistics software (IBM, Chicago, IL) and R package version 3.2.5. A nonparametric Mann-Whitney U test with Benjamini–Hochberg multiple testing correction was used, with FDR = 0.1, was used to analyse RNA editing data [22, 42]. We used specifically constructed R package scripts (available upon request). Analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) were used to analyze gene expression, isoforms distribution and behavioral data. LSD post-hoc tests were applied when interactions were significant. Means and SE are presented in the figures. Significance level was set at $P < 0.05$. Results that approach significance were defined as $.05 \leq P \leq 0.075$.

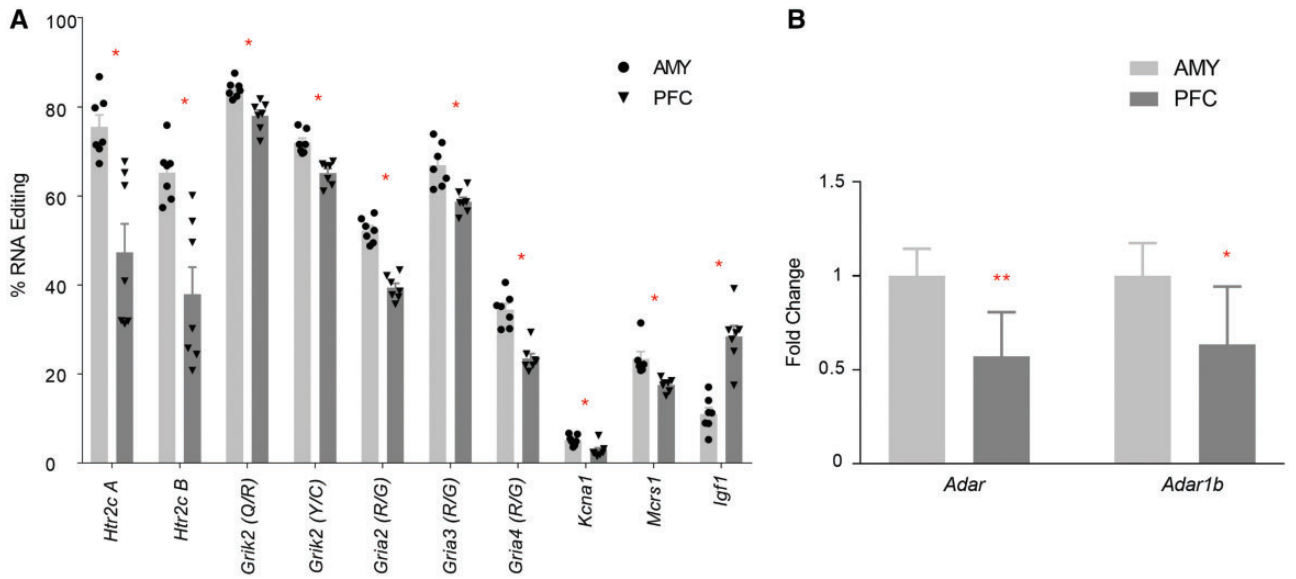

Figure 2: A-to-I RNA editing and ADAR gene expression in the neonatal PFC and amygdala. Significant differences in % RNA editing (**A**) and fold changes (means ± SE of the fold change relative to amygdala) in mRNA gene expression of editing enzymes (**B**) in the PFC and amygdala at P0. All samples are from offspring of control females treated with FLX prior to gestation. *$P < 0.05$. **$P < 0.001$. N's, RNA editing; PFC 7, amygdala 7; gene expression; *Adar* 7, 6; *Adarb1* 6, 7

Results

Editing Levels Are Lower in Neonatal PFC Compared with Amygdala

We previously showed that editing levels were generally lower in the neonatal rat PFC compared with the amygdala [22]. Here, we asked whether maternal pre-reproductive FLX treatment would affect these regional patterns. We detected editing at all 146 sites. Editing levels at 10 editing sites were different in PFC compared with the amygdala; in 9/10 sites, editing was lower in PFC (Mann–Whitney U test, FDR = 0.1; Fig. 2A; see Supplementary Table S1 for mean editing levels at each site in PFC and amygdala). mRNA expression levels of *Adar* and *Adarb1* were also lower in PFC (one-way ANOVAs, *Adar*, $F_{1,11} = 17.759$, $P = 0.0014$; *Adarb1*, $F_{1,11} = 8.788$, $p = 0.013$; Fig. 2B). There were no regional differences in *Htr2c* mRNA levels (NS, not shown).

Maternal Treatment with FLX and Exposure to Stress Prior to Pregnancy Affect RNA Editing Enzyme and *Htr2c* mRNA Expression at Birth

We previously showed that PRS affects mRNA expression levels of *Adar* and *Adarb1* (which encode ADAR1 and ADAR2, respectively) differentially in the PFC and amygdala of F1 offspring at P0. The mRNA expression of *Htr2c* (encoding the serotonin 5HT2C receptor) in offspring is also sensitive to maternal PRS [22]. Here, we asked whether maternal post-stress FLX treatment would reverse the effects of PRS, and/or have its own impact on expression levels. The effects of PRS on expression levels were previously reported [22] and are depicted in the gray panels in Fig. 3. Since FLX-exposed offspring samples were collected at the same time as non-exposed samples, we combined the data from the previous experiment [22] with the present one and applied a 2-way ANOVA analysis for each gene. We found that in the PFC (Fig. 3A), FLX increased *Adar* and *Adarb1* mRNA expression in offspring of control, but not PRS, dams, and reversed the PRS-induced increased in *Adarb1* [Fig. 3A1: *Adar*, main effect of group $F_{1,25} = 5.030$, $P = 0.034$, group × drug interaction $F_{1,25} = 8.528$, $P = 0.007$, post hoc C-FLX > C-VEH ($P = 0.017$); Fig. 3A2: *Adarb1*, group × drug interaction $F_{1,22} = 15.49$, $P = 0.0007$, post-hoc C-FLX>C-VEH ($P = 0.057$), PRS-FLX< PRS-VEH ($P = 0.001$)]. FLX also led to an increase in *Htr2c* mRNA expression in O-C, but not O-PRS, rats [Fig. 3A3, main effect of drug $F_{1,23} = 32.139$, $P = 0.000009$, group × drug interaction $F_{1,23} = 24.920$, $P = 0.0000476$, post-hoc C-FLX>C-VEH $P = 0.0003$)]. In the amygdala, we previously observed a PRS-induced decrease in the expression of RNA-editing enzymes and *Htr2c* in offspring of VEH-treated rats ([22], Fig. 3B *gray panels*). A similar effect of PRS was observed in offspring of FLX-treated rats (main effect of group, Fig. 3B1: *Adar*, $F_{1,20} = 3.757$, $P = 0.067$; Fig. 3B2: *Adarb1*, $F_{1,21} = 12.277$, $P = 0.0021$; Fig. 3B3: *Htr2c*, $F_{1,21} = 15.93$, $P = 0.00066$). In addition, FLX increased *Adar* (main effect Drug, $F_{1,20} = 26.548$, $P = 0.0000484$) and *Htr2c* expression (main effect drug, $F_{1,21} = 7.104$, $P = 0.014$) regardless of PRS exposure.

Maternal FLX Treatment Affects A-to-I RNA Editing at the *Htr2c*

Since maternal FLX treatment affected RNA editing enzyme expression in offspring PFC and amygdala at birth, independently of PRS effects, we examined the effects of FLX on A-to-I RNA editing levels in the same regions in stress-naïve (O-C) offspring. In the PFC, editing levels at the 146 detected sites were not affected by maternal FLX (Mann–Whitney U test, FDR = 0.1; see Supplementary Table S2 for mean + SE editing levels). In the amygdala, out of 146 detected editing sites we found FLX-induced differences only at the *Htr2c:* maternal FLX led to a decrease in editing at the A, B and C sites (Fig. 4; Mann–Whitney U test, FDR = 0.1; mean + SE data in Supplementary Table S2).

Supplementary Table S3 presents the change in % distribution of *Htr2c* isoforms in PFC and amygdala of O-C rats, excluding isoforms containing the E site where editing was not detected. As can be seen, there was no difference in distribution of *Htr2c* isoforms between VEH and FLX in PFC, but in the amygdala, the prevalence of 3 isoforms (VSV_{ABCD}, $F_{1,8} = 8.923$, $P = 0.0174$; VSI_{ABC}, $F_{1,8} = 6.611$, $P = 0.033$; VNI_{AB}, $F_{1,8} = 5\ 7.675$, $P = 0.024$), was significantly different in offspring of FLX-treated

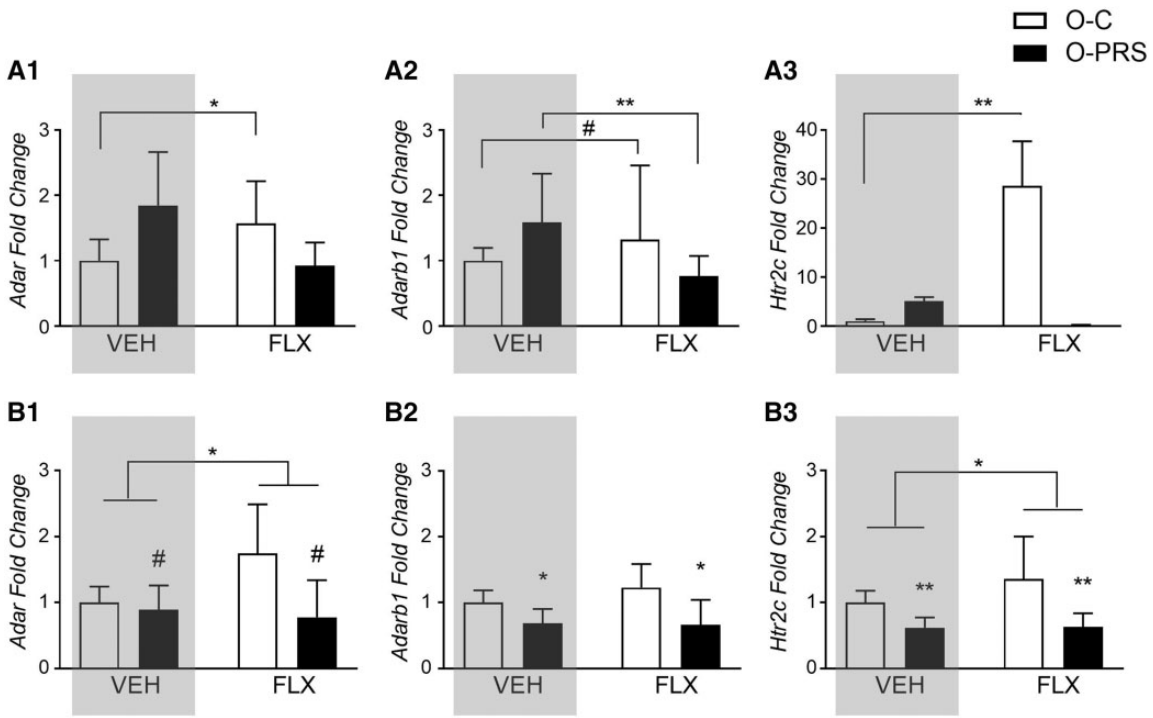

Figure 3: maternal PRS- and FLX-induced changes in *Adar*, *Adarb1* and *Htr2c* gene expression in the offspring brain. (**A**) Fold changes in mRNA gene expression of *Adar* (A_1), *Adarb1* (A_2) and *Htr2c* (A_3) in the PFC of offspring of PRS (O-PRS) and control (O-C) dams treated with VEH or FLX prior to gestation. (**B**) Fold changes in mRNA gene expression of *Adar* (B_1), *Adarb1* (B_2) and *Htr2c* (B_3) in the amygdala of offspring of PRS (O-PRS) and control (O-C) dams treated with VEH or FLX prior to gestation. Fold change values are presented as means ± SE of the fold change relative to O-C). #$P < 0.075$. *$P < 0.05$. **$P < 0.001$. Gray panels show previously published data [22]. N's, *Adar*, PFC: VEH, O-C 5, O-PRS 8, FLX, 6,7; amygdala: VEH, 4,9, FLX 7,5. *Adarb1*, PFC: VEH, O-C 5, O-PRS 8, FLX, 7,7; amygdala: VEH, 4,8, FLX 7,6. *Htr2c*, PFC: VEH, O-C 6, O-PRS 8, FLX, 7,8; amygdala: VEH, 4,8, FLX 6,6.

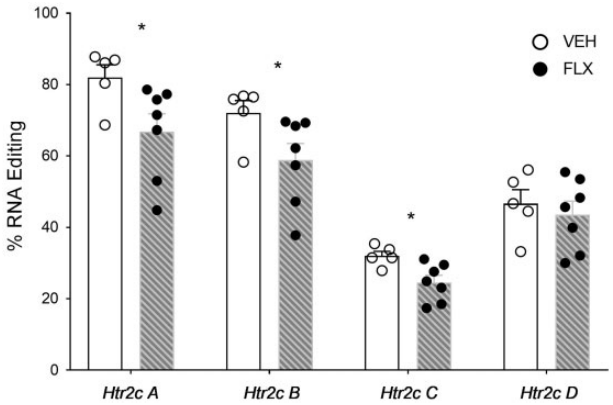

Figure 4: maternal FLX treatment-induced changes in A-to-I RNA editing in offspring amygdala at birth. Percent RNA editing at the *Htr2c* A–D sites in the amygdala of neonatal offspring of rats treated with VEH or FLX prior to reproduction. *$P < 0.05$. N's, VEH 5, FLX 7

compared with VEH-treated dams. Notably, prevalence of the highly edited isoform VSV_{ABCD} was decreased in offspring of FLX-treated dams.

Maternal FLX Treatment Can Reverse or Enhance the Effects of PRS on A-to-I Editing in the PFC

We next asked whether maternal FLX treatment would reverse some of the effects of PRS on editing in offspring PFC and amygdala. Previous analysis of multiple sites where editing affects neurotransmission and the stress response (29 sites in total; [22]), revealed significant PRS-induced changes in editing at *the Htr2c* in PFC [22]. Here, we assessed the difference in editing levels between O-PRS and O-C samples at each of these sites, and asked whether maternal FLX treatment would alter this difference. Figure 5 presents sites where the editing in O-PRS samples was significantly different from O-C samples (Mann–Whitney analysis, FDR = 0.1), in either VEH or FLX groups. In the PFC (Fig. 5A), PRS led to significant changes in editing at the A and B sites of the *Htr2c*, and FLX reversed this effect. However, FLX treatment enhanced the effect of PRS on editing at mRNA encoding AMPA and KA glutamate receptor subtypes, leading to differences between O-PRS and O-C groups that were not present in VEH samples. In the amygdala (Fig. 5B), PRS led to significant changes in editing at mRNA encoding serotonin and glutamate receptors. FLX reversed the effects of PRS at some of the sites and left differences intact at others. Unlike in the PFC, FLX did not enhance the difference between O-PRS and O-C samples at any of the sites examined (see Supplementary Table S4 for full results).

Supplementary Table S5 presents the difference in *Htr2c* isoform prevalence between O-PRS and O-C samples, separately for VEH- and FLX-treated groups (differences between VEH-treated O-PRS and O-C groups were previously published [22]). As can be seen, FLX treatment affected the prevalence of isoforms in offspring PFC and amygdala in interaction with the effects of PRS. Particularly notable is the effect of FLX on the prevalence of the INI isoform (no editing at any of the *Htr2c* sites) in the PFC: whereas PRS had no effect on its prevalence in VEH-treated samples, it increased its prevalence by 22% when dams were treated with FLX.

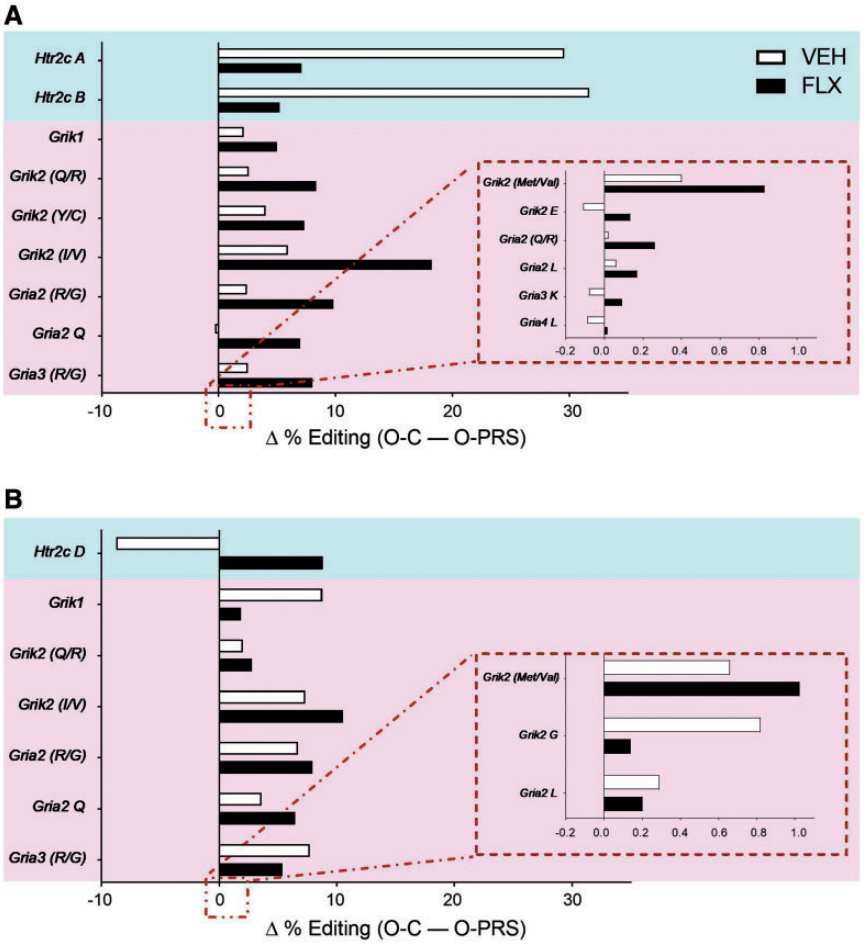

Figure 5: maternal FLX-induced modulation of PRS effects on RNA editing levels in offspring PFC. Significant differences in RNA editing in the PFC (**A**) and amygdala (**B**) between O-C and O-PRS rats are presented as delta % editing. Dams were treated with VEH or FLX prior to reproduction. Blue panels highlight editing changes at *Htr2c*. Pink panels highlight editing changes in glutamate receptor subunits. *Insets:* Editing sites where editing differences were between −0.2 and 1. **N's**, PFC: VEH, O-C 5, O-PRS 8; FLX, 7, 5; amygdala: VEH, 5, 9; FLX, 7, 6

Maternal FLX Treatment Leads to Enhanced Social Preference in Adult Offspring

Since FLX affected RNA editing and gene expression patterns in offspring independently of stress exposure, particularly at mRNA encoding glutamate receptors (Fig. 5), we examined the effect of pre-reproductive FLX treatment in interaction with PRS on social preference (Fig. 6A). Social preference is a task that measures the animal's tendency to preferentially explore an unfamiliar social stimulus versus an unfamiliar inanimate object, and relies on intact NMDA receptor-mediated glutamate neurotransmission in the PFC [48, 49]. We first compared the performance of male and female rats in the task, and found that exploration duration of the social stimulus was higher in females (mean = 116.350, SE = 4.677) compared with males (mean = 94.156, SE = 4.284; main effect of sex $F_{1,88}$ = 12.434, $P = 0.00067$). No differences in non-social stimulus exploration time were found (NS; N's: Females; VEH, O-C 13 O-PRS 10, FLX, O-C 17 O-PRS 10).

A separate analysis of FLX and PRS effects in males and females revealed that male, but not female, offspring were affected by pre-reproductive FLX treatment (Fig. 6, data for females not shown). A two-way ANOVA analysis of partner exploration time revealed a significant main effect of drug $F_{1,42}$ = 4.488, $P = 0.04$, and a group × drug interaction $F_{1,42}$ = 6.230, $P = 0.0165$. Post-hoc analysis revealed that maternal FLX treatment increased partner exploration time in O-C animals (O-C/FLX>O-C/VEH, $P = 0.0043$), but not in offspring of rats exposed to stress (O-PRS/FLX< O-C/FLX, $P = 0.0733$).

Discussion

The present study shows that treatment of female rats with the antidepressant drug FLX prior to reproduction and gestation affects RNA editing patterns, gene expression of editing enzymes and behavior in offspring. Pre-gestational FLX modulates some of the effects of prior exposure to stress, but also produces independent consequences. These findings have implications on current understanding of serotonergic signaling and its sensitivity to maternal exposure to stress and antidepressant drug treatment.

Pre-gestational treatment of adolescent female rats with FLX affects RNA editing patterns in their offspring at birth. FLX alters serotonergic transmission by blocking the serotonin transporter and increasing synaptic serotonin levels [50]. Although in our study FLX treatment was discontinued a week prior to gestation, a direct influence of the drug on the developing fetus cannot be ruled out, as previous studies have shown that FLX and particularly its active metabolite, norfluoxetine,

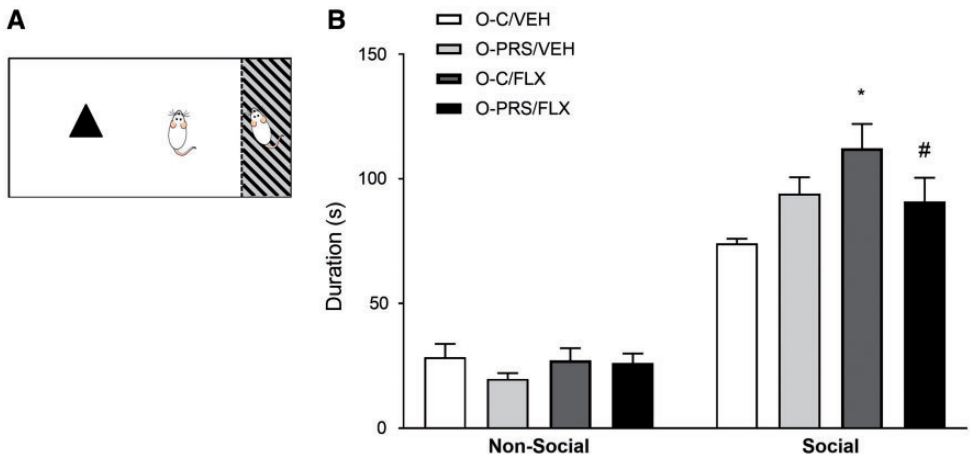

Figure 6: FLX maternal PRS- and FLX-induced changes in social preference in adult male offspring. (**A**) A representation of the social preference behavioral paradigm. Rats explore a novel inanimate (non-social) or a social stimulus for 5 min. (**B**) Time spent exploring social and non-social stimuli in male adult offspring of control (O-C) or stressed (O-PRS) dams treated with VEH or FLX. No differences in non-social stimulus exploration were found (NS). *$P < 0.05$. N's, VEH, O-C 8, O-PRS 16; FLX, 11, 11

have relatively long elimination half-lives and can remain in the plasma after drug administration is discontinued [51, 52]. FLX and other SSRIs cross the placenta, enter fetal brain tissue and are present in breast milk [53–56]. Gestational exposure to FLX was shown to induce age-specific and region-specific alterations in serotonin levels and receptor densities in offspring brain [57–59]. We showed that pre-gestational exposure to FLX increased mRNA expression of *Htr2c* (Fig. 3), and selectively altered editing at this receptor (Fig. 4) while leaving editing at 142 non-serotonergic sites intact. These alterations could be due to a direct interaction of FLX with developing serotonergic signaling pathways, or to indirect effects of FLX on the developing fetus which give rise to editing and gene expression changes in the neonatal brain. Another possibility is that serotonin reuptake inhibition directly affects oocytes, leading to changes in offspring gene expression patterns. Serotonin and elements of a regulatory serotonergic system are present in oocytes and may be modified by FLX treatment [60]. We previously showed that PRS alters the expression of stress-related corticotropin releasing hormone receptor type 1 (CRFR1) in oocytes and in offspring brain at birth [31]. We did not find editing changes in stress-exposed oocytes [22], but FLX-induced changes in editing, editing enzymes and molecules that regulate serotonergic activity in oocytes should be the subject of future exploration.

Previous studies have shown that the expression of ADAR enzymes and A-to-I RNA editing at sites encoding serotonin, glutamate and GABA receptors are altered by FLX treatment [17, 33–35, 37, 61]. Curiously, here we found that maternal treatment with FLX led to opposite effects on *Htr2c* editing and ADAR mRNA expression levels in offspring: whereas editing at the *Htr2c* A and B sites decreased, mRNA expression of editing enzymes increased. We and others [22, 23, 62–67] previously found that changes in editing levels correlated poorly with ADAR mRNA or protein expression levels. Differences between mRNA and protein expression or between expression and activity levels, compensatory or self-regulatory mechanisms could account for the non-linear relationship between editing rates and editing enzyme expression levels [68]. Furthermore, ADAR enzymes regulate additional processes, e.g. miRNA biogenesis and function [68], which could be affected by PRS and/or FLX.

Interestingly, we found that editing rates as well as ADAR mRNA expression levels in offspring of FLX-treated rats were higher in the amygdala compared with the PFC (Fig. 2), in line with our previous findings in offspring of VEH-treated rats [22] and with the maturational profile of these regions [48, 49]. This positive correlation could be due to the fact that regional differences in editing were not limited to the *Htr2c*, as were FLX-induced effects.

Htr2c editing affects the expression and activity levels of this G-protein-coupled receptor, so that increased editing generally results in reduced sensitivity to ligands, reduced basal activity [15, 69], decreased G-protein coupling [70] and decreased intracellular signaling [71]. Here, maternal FLX treatment decreased A and B site editing (Fig. 4) and increased *Htr2c* mRNA levels (Fig. 3) in the amygdala, and these effects were accompanied by decreased prevalence of the unedited INI isoform and increased prevalence of the highly edited (and presumably less functional) VSV isoform (Supplementary Table S3). Notably, the very pronounced FLX-induced increase in *Htr2c* mRNA expression in the PFC (>28-fold) was not accompanied by changes in receptor editing. While the present study does not enable us to determine whether changes in editing enzyme or substrate expression preceded changes in editing, one possibility is that maternal FLX treatment affected serotonergic signaling in the developing fetus and led to increased *Htr2c* mRNA expression in neonate offspring, which in turn impacted *Htr2c* editing rates and editing enzyme levels.

Pre-gestational, post-stress treatment of rats with FLX reverses some of the effects of PRS on the mRNA expression of RNA editing enzymes in offspring brain. Chronic unpredictable stress in adolescence, a vulnerable time period in neural development, is associated with the emergence of psychiatric disorders in adulthood [72]. FLX and other SSRIs are the most frequently prescribed anti-depressants, and are increasingly prescribed in adolescence and during pregnancy [73–76]. Chronic unpredictable stress is a rodent model of depression and anxiety [77], and FLX as well as other SSRIs were shown to reverse the effects of stress on depression-like behavior and HPA axis function in rodents [78]. While this is the first study to investigate the interaction between PRS and pre-gestational FLX exposure, several studies examined the consequences of perinatal FLX exposure in rodent models of depression. For example, a recent study showed that perinatal treatment (from Gestational Day 10-P21) with FLX reversed pre-gestational stress-induced abnormalities in serotonin levels and turnover in offspring PFC [79]. Other studies showed that perinatal FLX

can reverse the effects of maternal stress on immobility in the forced swim test, hippocampal neurogenesis, and 5-HIAA levels in the hippocampus of juvenile or adolescent offspring [79–81].

Similarly, in the present study pre-gestational treatment with FLX reversed the consequences of PRS on editing abnormalities at the *Htr2c* in PFC and amygdala of offspring. Our results indicate that discontinuation of treatment prior to pregnancy does not alter FLX effects. As discussed above, this may be due to the long half-life of FLX and its metabolites and its ability to cross the placenta and enter fetal brain tissue, or to direct effects on serotonin dynamics in exposed oocytes. FLX and other SSRIs were hypothesized to exert their antidepressant actions by normalizing hypothalamic-pituitary-adrenocortical (HPA) system hyperactivity, a central clinical feature of depression. Salari *et al.* [81], for example, found that the effects of gestational stress on corticosterone elevations in mice were reversed by either gestational or perinatal (P10-20) FLX treatment. However, in other studies FLX treatment during gestation or in adult rodents potentiated HPA system hyperactivity on its own rather than reducing the effects of stress [81–85].

The 5-HT2C receptor is implicated in the response to chronic stress and maintains reciprocal relations with the HPA system [86, 87]. Chou-Green *et al.* [88] found that 5-HT2cR knockout mice are hyper-responsive to stress, and others have shown that stress alters *Htr2c* editing patterns. Both elevated and reduced editing levels can have deleterious effects on behavior. For example, mice expressing either the fully edited or fully unedited form of the 5-HT2c exhibit anxiogenic behavior, particularly in the BALB/c strain commonly used as a mouse model of anxiety and depression [89, 90]. In our study, FLX increased the proportion of the unedited (INI) isoform in offspring of PRS-exposed, but not control rats (Supplementary Table S5). In the context of previously published literature, our findings imply that FLX treatment leads to increased signaling at the 5-HT2c receptor particularly when the HPA axis is activated.

While reversing the effects of PRS on editing at the *Htr2c*, FLX treatment led to enhanced differences between C and PRS-exposed offspring at glutamate receptors, particularly in the PFC (Fig. 5). Disrupted glutamatergic neurotransmission is implicated both in the pathogenesis of affective disorders and in the outcome of pharmacological treatments [91–94]. Editing of AMPA and KA receptor subunits was shown to have a significant effect on transmission dynamics (e.g. [11, 95, 96], and is sensitive to environmental manipulations, e.g. learning and stress [23, 97]. In our hands, pre-reproductive chronic unpredictable stress did not affect editing at glutamate receptors in affected female rats or their offspring [22]. In line with previous studies that showed minor effects of FLX on glutamate receptor editing [98], in the present study we showed that pre-gestational FLX treatment on its own also had no effect on editing in newborn offspring. However, FLX treatment potentiated differences in editing of glutamate receptors between offspring of control and stress-exposed rats. This interaction between stress and drug effects seemed to be specific to glutamate receptors, was particularly pronounced in the PFC, and encompassed different editing sites at AMPA and KA receptors (Fig. 5, Supplementary Table S4). This finding may support the interaction between serotonergic and glutamatergic transmission in the modulation of stress effects, and provide a possible mechanism for plasticity in response to environmental adversity.

Deficits in social function are shared by several psychiatric disorders, including autism, depression and schizophrenia [99]. Treatment with SSRIs, including FLX, ameliorates social deficits in depressed and anxious individuals [100], and reverses aberrant social behavior in animal models of psychopathology (e.g. [101]). Here, we found that pre-gestational treatment with FLX-enhanced social preference in adult male, but not female, offspring (Fig. 6). Assuming that pre-gestationally administered FLX had a direct effect on the developing fetus, this finding can be explained by the known effects of perinatal FLX administration on the developing serotonergic system [102]. A sexually dimorphic effect of pre- or perinatal SSRI exposure on social behavior has been previously reported (see [103] for review). For example, Svirsky *et al.* [104] found that prenatal FLX increases aggression in male offspring and delays the onset of maternal behavior in females. Interestingly, in our study the effect of FLX on social behavior was attenuated in offspring of stress-exposed females. In agreement with this finding, exposure to prenatal stress diminished the prenatal FLX-induced increase in social (aggressive) behavior in male offspring [105]. The mechanism for this stress × drug interaction on social behavior in offspring remains to be elucidated, but may be related to opposite effects of PRS and FLX on the HPA axis. Furthermore, the interaction between PRS and FLX effects on measures of anxiety and cognitive behavior are to be explored in future studies. Finally, a relationship between FLX-induced changes in *Htr2c* RNA editing or expression levels at birth and FLX-induced changes in adult social behavior cannot be deduced from this study but may provide important clues for the role played by intact development of the serotonergic system in early life and social function in adulthood.

In summary, this is the first study to investigate the effects of pre-gestational treatment with FLX on offspring, as most human and animal model investigations focus on the effects of SSRI treatment during pregnancy. We found that FLX administration to female rats prior to gestation affected 5-HT2C receptor expression and editing in neonatal offspring brain, led to enhanced social preference in adult offspring, and interacted with the effects of prior exposure to stress. Chronic unpredictable stress is commonly used to model depression in humans, and FLX is often the first line of treatment for stress-related depression in adolescents and during pregnancy. Here, we demonstrate that even when discontinued prior to gestation, FLX has long-lasting effects on serotonin dynamics and on social behavior.

Supplementary Data

Supplementary data are available at *EnvEpig* online.

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